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14. ABSTRACT

The plasminogen cascade of serine proteases has been affiliated in the mammary gland withboth development and tumorigenesis. We have found that the dominant plasminogen activatorduring mammary gland stromal involution is plasma kallikrein (PKal), and that active PKalappears in connective tissue-type mast cells in the mammary stroma during different phases ofdevelopment. Examination of the extrahepatic expression of PKal has shown that PKal messageis present in the mammary gland, and that increased expression levels correlate to periods ofstromal remodeling. Additionally, an inhibitor of PKal that has been demonstrated to diminishmammary gland involution may be used to characterize PKal expression in the mammary gland aswell as to identify targets of PKal activity during involution. Furthermore, mast cells arerequired for normal mammary duct branching morphogenesis during puberty. Lastly, a PKalknockout mouse has been produced that has the unexpected consequence of embryonic lethality.

15. SUBJECT TERMS
mammary gland, serine protease, plasma kallikrein, mast cell, mouse model, involution,adipocyte, extracellular matrix, plasminogen

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INTRODUCTION

The plasminogen cascade of serine proteases has been affiliated in the mammary gland with both development and tumorigenesis. The ultimate effector in this cascade, plasminogen (active form: plasmin), is managed by an intricate cascade of plasminogen activators and protease inhibitors. Plasminogen activators and inhibitors are strongly associated with poor prognosis in a variety of human tumors, including breast cancer. [1] Furthermore, plasminogen-deficient mice yield significantly fewer metastases in a viral oncogene-induced model of breast cancer[2, 3] and exhibit significant defects in lactational competence and post-lactational mammary gland involution.[4] This study focuses on the role of a plasminogen activator in murine mammary stromal development and metastasis. This lab has demonstrated that the dominant plasminogen activator for mammary stromal involution is plasma kallikrein (PKal)[5] and that active PKal appears in connective tissue-type mast cells in the stroma and surrounding the blood vessels of the murine mammary gland. This project aims to study the plausible relationship between mast cell activation, PKal release, and plasmin conversion in the mammary gland by examining the role of PKal in mammary gland development, involution and metastasis. This report details the work accomplished during the second year of my predoctoral traineeship award.

BODY

Task 1. The generation of a prekallikrein-deficient mouse is the crucial first step towards determining the effects of the loss of PKal on mammary gland development and tumorigenesis. Prekallikrein consists of two domains: a binding domain, and a trypsin-like serine protease domain (Figure 1). Its binding domain, consisting of four apple (PAN) domains, is thought to mediate interactions between prekallikrein and plasma-borne PKal activators such as Factor XII.[6, 7] PKal's serine protease domain is highly conserved amongst other proteases in the plasminogen cascade as well as other members of the large trypsin-like protease family. The last annual report detailed the reasons for the modification of the approved Statement of Work concerning Task 1.

Task 1a. As previously reported, cloning difficulties led to a delay in the generation of the PKal (allele name: *Klkb1*) knockout construct, and then screening of E14 (SV129/Ola) embryonic stem (ES) cell colonies for correct targeting by Southern blot hybridization also proved challenging. In month 2 of this second award period, three ES cell lines confirmed to be correctly targeted were analyzed by karyotype, and two were found to be sufficiently normal (>90% of cells typed had normal chromosome number) to be suitable for blastocyst injection. The *Klkb1* targeting strategy is depicted in Figure 2.

Task 1b. Blastocyst injections of two clones, D10 and E8, were performed in months 3 and 4 by the UCSF Comprehensive Cancer Center Transgenic/Targeted Mutagenesis Core. Three male chimeras from the E8 line were obtained from the first round of injections, and two male chimeras from the D10 line resulted from the second round.

Task 1c. Upon reaching sexual maturity, chimeras were mated with C57Bl/6 females to generate heterozygous offspring. The first F1 generations were born in month 7 of this award period. Two of the three E8 chimeras yielded consistent offspring and demonstrated germline transmission of the *Klkb1*^{tm1} allele. The third E8 chimera only yielded two female offspring and never successfully mated thereafter. The two D10 chimeras completely failed to generate offspring; it is likely that they were phenotypic males, without functional gametes, as is common in chimeras obtained through blastocyst injection.[8] Regardless, sufficient numbers of F1

progeny from the functional E8 chimeras were determined by Southern blotting and by polymerase chain reaction (PCR) to be heterozygous to confirm germline transmission and therefore set up heterozygous mating pairs. Assessment of heterozygous animals for GFP expression showed that GFP expression by immunofluorescence was consistent with that of plasma kallikrein by *in situ* hybridization (Figure 3). Therefore, GFP will be used as a reporter of PKal expression; though endogenous GFP fluorescence appears weak even in the liver, where PKal is largely expressed, visualization via immunofluorescence works well.

Task 1d. Heterozygous F1 *Klkb1*$^{+/tm1}$ mice did not exhibit any overt phenotype and proved to be fertile and lactationally competent. F2 progeny, representing offspring from heterozygous breeding couples, were first generated in month 10 of this award period. To date, nineteen F2 litters have been generated, and no live homozygous mutant animals have been identified. Wild-type and heterozygous mice appear in nearly all litters, and of 134 progeny from these nineteen litters, 39 were wild-type and 95 were heterozygous, in a ratio of approximately 2.4:1. The likelihood of this result as non-Mendelian (expected 1 wildtype: 2 heterozygotes: 1 homozygote mutant) is $p = 0.97x10^{-12}$ as determined by a χ^2 test for goodness-of-fit. As this was strongly suggestive of an embryonic lethal homozygous phenotype, work was begun in the last six weeks of this award period to identify homozygous mutants *in utero*. F2 litters from heterozygous crosses have been analyzed at embryonic day (E) 12, 10.5, 9.5, 8, and 7.5. At E12, 10.5, and 9.5, no homozygous mutants were genotyped. At E8 and E7.5, sufficient genomic DNA has not been recovered to perform genotyping by PCR; however, abnormal embryos have been observed at these timepoints (Figure 4). It is yet to be determined whether these abnormal embryos are due to loss of PKal or the result of natural attrition; further analysis is underway.

<u>*Task 2*.</u> Analysis of plasma kallikrein expression in the mouse mammary gland is necessary to confirm preliminary data suggesting extrahepatic expression of PKal in the mammary gland stroma and/or in connective tissue-type mast cells. If plasma kallikrein is produced outside of the liver in tissues that require its activity and is activated non-canonically apart from the contact activation system in blood vessels, then this would represent a novel pathway for the plasminogen cascade of protease activity. It has recently been shown that plasminogen is expressed by a wide range of tissues [9] therefore, it is not unreasonable to hypothesize that tissues in which plasmin activity is required would also have locally expressed plasminogen activators to better control the activation cascade.

Task 2a. Plasma kallikrein expression in the mouse mammary gland at different developmental time points (3 weeks, 5 weeks, 15 days pregnant, 10 days lactating, and 4 days involuting) was assessed using real-time PCR. As reported in the last annual summary, and shown in Figure 5, prekallikrein message is present strongly during virgin development, when the mammary gland is undergoing active remodeling as the ductal epithelium expands and advances through the stromal fat pad. During pregnancy and lactation, when the stroma has largely been replaced by secretory alveoli and extensive ductal structures, prekallikrein message is markedly reduced. Prekallikrein message increases significantly during the program of mammary gland involution, when the secretory lactation structures apoptose and the mammary stromal compartment is replenished. These findings confirm that not only is PKal produced in the mammary gland, but that increased expression levels correlate to periods of stromal remodeling.

Task 2b. Using ecotin PKal, a macromolecular inhibitor of active PKal [10], we have shown that inhibition of PKal during mammary gland involution significantly inhibits adipocyte

replenishment and stromal remodeling (J. Lilla, unpublished data). To confirm that the target of this inhibitor *in vivo* is PKal, and to determine whether other factors are affected by ecotin PKal, I have collected mammary gland lysates from different stages of development to be analyzed for binding partners to ecotin PKal. Analysis was initially delayed, as the experimental plan was dependent on the availability of prekallikrein-deficient mice generated from the first set of tasks (see above) to serve as an essential negative control for this set of experiments. However, in anticipation of the possibility that such controls will not be available due to the unexpected unavailability of viable null animals, some preliminary pull-down experiments have been performed, using a biotinylated inhibitor of active PKal (ecotin PKal, or EcoPK) and mammary gland lysates from virgin, 5 week-old mice. As indicated in Figure 6, EcoPK-coated streptavidin beads pull down plasma kallikrein from mammary tissue lysates. The bands indicated on the blot represent the full-length protein (appx. 80 kDa), and its heavy (42 kDa) and light (28 kDa) chains, both of which have likely glycosylation sites. Further refinement of this pull-down assay is necessary, so that the technique may be applied to mast cell culture lysates and/or mast cells recovered from peritoneal lavage to determine whether mast cells express PKal as indicated by previous staining (J. Lilla, data not shown).

Task 2c. As inhibition of PKal retards adipocyte replenishment and stromal remodeling during mammary gland involution, it is important to determine whether this inhibition is due to impaired plasminogen activation by PKal, or if there are other downstream targets of PKal during involution. Plans to address this question were once again impaired by the delay in obtaining prekallikrein-deficient mice, which would serve as an essential control for this analysis. Alternative methods may be considered, and planned microarray experiments may have to proceed in the next award year without benefit of the PKal knockout mouse.

Task 2d. It has been demonstrated previously that inflammatory cells play an important role in both mammary gland development [11, 12] and mammary tumor progression [13-16]. However, no previous work has attempted to elucidate the specific role of mast cells in mammary gland development. As shown in Figure 7, mast cells are present throughout early postnatal development, as well as during lactation and post-lactational involution. For the last three months of this reporting period, mammary development has been analyzed in the W-*sash* ($Kit^{W\text{-}sh/W\text{-}sh}$) mouse on the C57Bl/6 background, which is deficient in connective tissue-type mast cells.[17, 18] While prepubertal mammary glands do not seem to differ in the extent of ductal elongation, duct branch number, or number of terminal end buds (TEBs, the advancing edge of a branching duct) at 3 weeks of age, by 5 weeks, there are significant differences between W-*sash* mutants, heterozygotes, and their wild-type littermates. At 5 weeks, the number of TEBs in the ductal invasion front of W-*sash* mutants and heterozygotes is reduced somewhat as compared to their wild-type littermates as determined by ANOVA ($p=0.0885$), and the number of total duct ends is significantly diminished ($p=0.0006$) (Figure 8). This suggests that mast cells are necessary for proper formation of the invading ducts during mammary branching morphogenesis. Additionally, at 5 weeks of age, ductal penetration is significantly impaired ($p=0.0049$), positing mast cells as facilitators of duct elongation (Figure 9). To date, no significant differences have been observed in the ability of W-*sash* mice to undergo post-lactational involution, though this may be a background-dependent effect (J. Lilla, unpublished data). In the next several months of this award, W-*sash* mammary development analysis will be completed, followed by a series of experiments to confirm that reintroduction of mast cells rescues the effect of mast cell deficiency during mammary development. In addition, experiments will be performed to determine whether inhibition of PKal exacerbates or does not affect the function of mast cells during mammary

development.

KEY RESEARCH ACCOMPLISHMENTS

- A PKal mutant mouse line has been established, and may reveal the novel and unexpected finding that prekallikrein loss results in embryonic lethality.
- Prekallikrein message is present in the mammary gland, and increased levels correlate to phases of stromal remodeling.
- Ecotin PKal, a macromolecular inhibitor of plasma kallikrein, can be used to "pull-down" PKal from mammary tissue lysates.
- Mast cells affect mammary ductal morphogenesis during puberty.

REPORTABLE OUTCOMES

None to date.

CONCLUSION

Earlier delays to the generation of the PKal knockout mouse were overcome, and the mouse line was generated during the second year of this award. The surprising result that loss of PKal may lead to embryonic lethality will obviously hinder planned experiments that depended on viable adults to provide kallikrein-deficient tissues for analysis of mammary development, and ultimately, the effect of PKal on breast cancer development. If PKal-null embryos are dying around E7.5-8, this would suggest that PKal is involved in very early vascular events such as formation of blood islands and the embryonic yolk sac: a finding that would likely be tied to PKal's well-described physiological role in bradykinin activation.[7, 19-21] It is important to note that prekallikrein deficiency (Fletcher trait) in humans does not result in lethality [22]; however, the extent of the phenotype has not been fully characterized, i.e., it is not known if Fletcher factor deficiency represents a total loss of prekallikrein transcription, a truncated or absent protein product, or a functional mutation. Further analysis of the PKal line generated during this research should provide further insights into its elementary physiological and developmental roles.

Plasma kallikrein activity appears to be associated with the function of two major stromal cell types of the mammary gland: mast cells and adipocytes. This research aims to elucidate the role of PKal in mammary gland involution and metastasis. As many models of breast cancer highly implicate stromal signals as effectors or inhibitors of breast cancer progression, it is imperative to acquire a better understanding of the normal functioning of this protease to prepare for its possible characterization as a breast cancer indicator like other members of the plasminogen protease cascade, or as a target for drug therapies. And as many breast cancers are strongly associated with mast cell infiltration (in addition to other leukocytes), it is important to understand their normal physiological role in breast development, perhaps as mediators of protease activity and as attractors to other inflammatory cells.

The work described in this report has not only addressed interesting questions as to the function and importance of previously little-regarded participants in mammary gland biology, plasma kallikrein and mast cells, but has required the investigator to master a wide-reaching and comprehensive set of experimental techniques, from molecular biology to biochemistry to tissue biology and to animal husbandry. This wide range of skills should prove indispensable to a future career as a breast cancer researcher. In addition, this course of study has required contact

with related disciplines, such as research in wound healing, skin cancer, and prostate cancer, all of which are important as parallels in those fields are often made to breast cancer, to the benefit of all. It is the hope of the investigator that by the conclusion of this predoctoral award, she will be well-prepared to pursue many more avenues of fruitful investigation of breast cancer.

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APPENDICES

None.

SUPPORTING DATA

Figure 1. Domains of Plasma Kallikrein
Prekallikrein (active form: Plasma Kallikrein) consists of 4 apple (PAN) domains and a trypsin-like serine protease domain (catalytic residues H-D-S). Activation of the protease requires cleavage between the heavy and light chains by Factor XII.

Figure 2. Gene targeting strategy for *Klkb1*^{tm1}. EGFP was inserted immediately after the endogenous Klkb1 ATG. Recombinants were screened by Southern blot for presence of the hygromycin resistance gene using five different restriction enzymes outside of the sites of homologous recombination: *Bgl*II, *Spe*I, *Eco*RV, *Pvu*II, and *Stu*I.

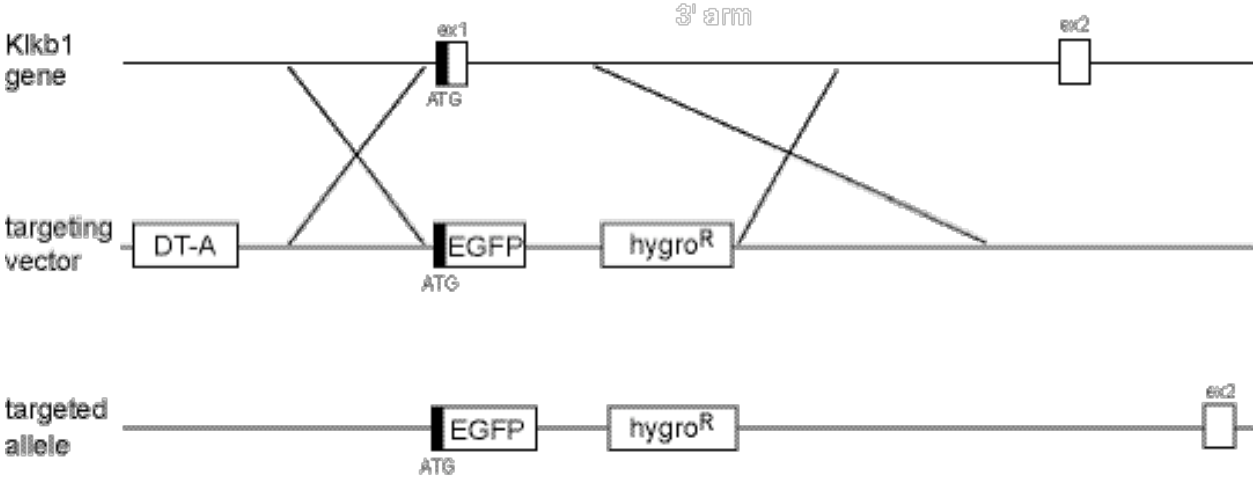

Figure 3. Expression of GFP in a *Klkb1*$^{+/tm1}$ mouse appears identical to localization of prekallikrein message in the liver. A) *In situ* hybridization for prekallikrein in a wild-type mouse liver, 100x. B) Immunofluorescence using anti-GFP antibody on *Klkb1*$^{+/+}$ and *Klkb1*$^{+/tm1ZW}$ littermate liver, 200x.

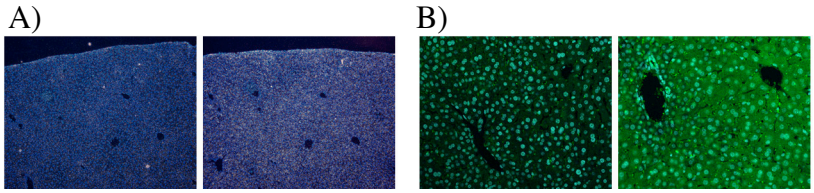

Figure 4. F2 Embryos at embryonic day 7.5 recovered from a *Klkb1* heterozygous cross. At left, abnormal embryos appear much smaller, and lack distinct embryonic-extraembryonic boundaries. The ectoplacental cone is also significantly diminished.

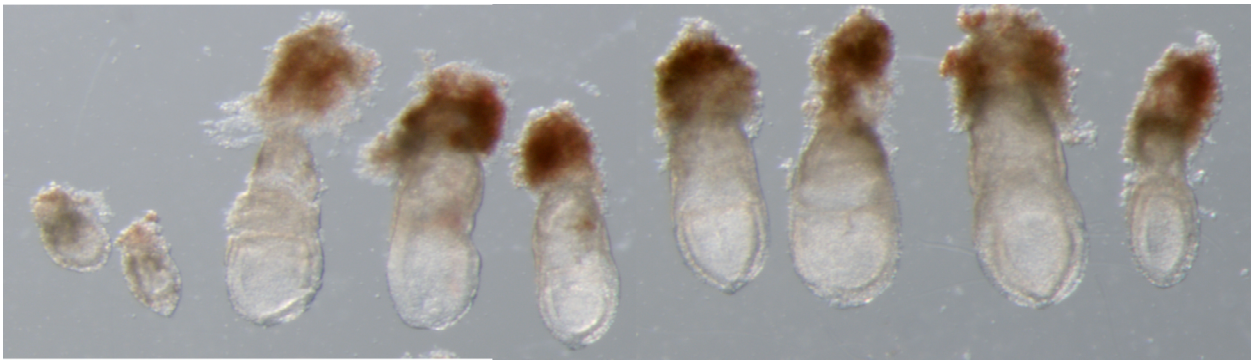

Figure 5. Real-time PCR analysis of plasma kallikrein expression in mammary gland RNA

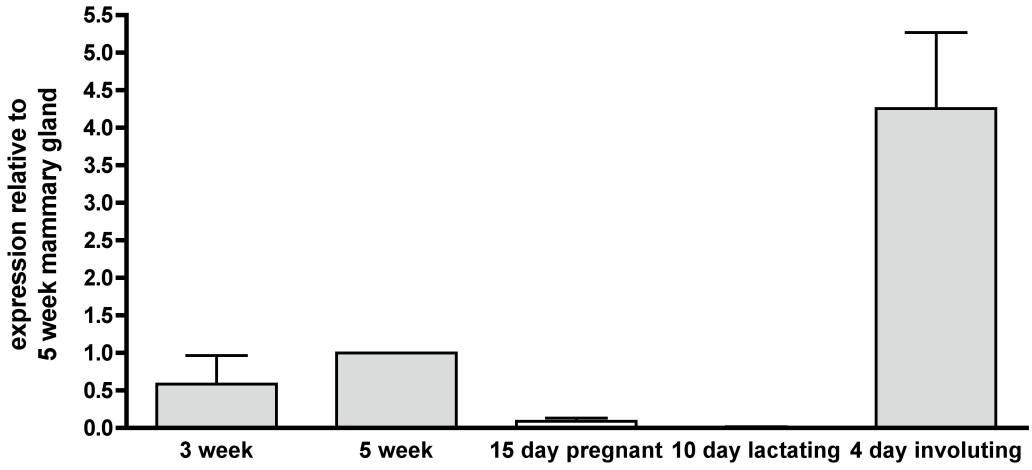

Figure 6. Ecotin PKal binds plasma kallikrein isolated from mammary gland lysates. Protein lysates from five week old virgin mammary glands were run over either streptavidin beads or beads bound with biotinylated EcoPK ("pull-down") and subjected to Western blotting using an antibody against mouse prekallikrein (α-mouse KLKB1, R&D Systems). The bands likely represent full-length PKal (appx. 80 kDa), its heavy chain (42 kDa), and its light chain (28 kDa, unglycosylated). mPKal = mouse prekallikrein peptide, against which the antibody was raised.

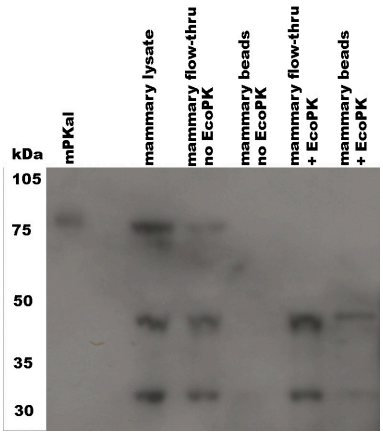

Figure 7. Mast cells are present throughout postnatal mammary gland development. Naphthol Chloroacetate Esterase staining of frozen mammary gland sections, all 160x. Mast cells granules are stained light red, nuclei are blue (Gill's hematoxylin #2 counterstain). Note that mast cells are found in the fatty stroma, but primarily around ducts and blood vessels.

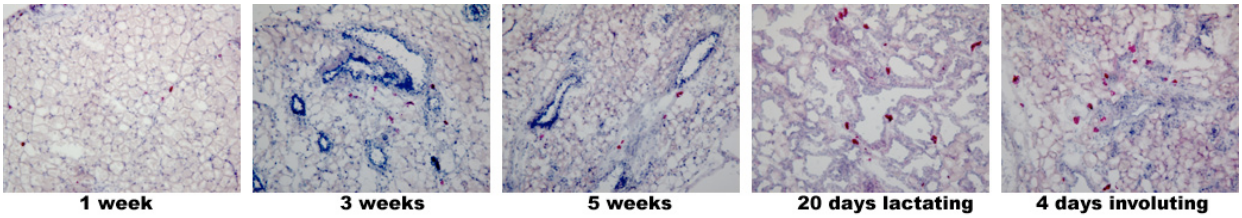

Figure 8. Mast cells affect formation of terminal end buds and duct branch number in pubertal mammary gland development. Mast cell deficient mammary glands have fewer invasion front terminal end buds (TEBs) and fewer invasion front duct ends than their W-*sash* heterozygous littermates at 5 weeks of age. ANOVA for duct ends: p=0.0006 at 5 weeks of age. Whole mounts of left inguinal mammary glands were collected and examined for TEB number and duct end numbers past the midline of the inguinal lymph node (marking the invading edge boundary).

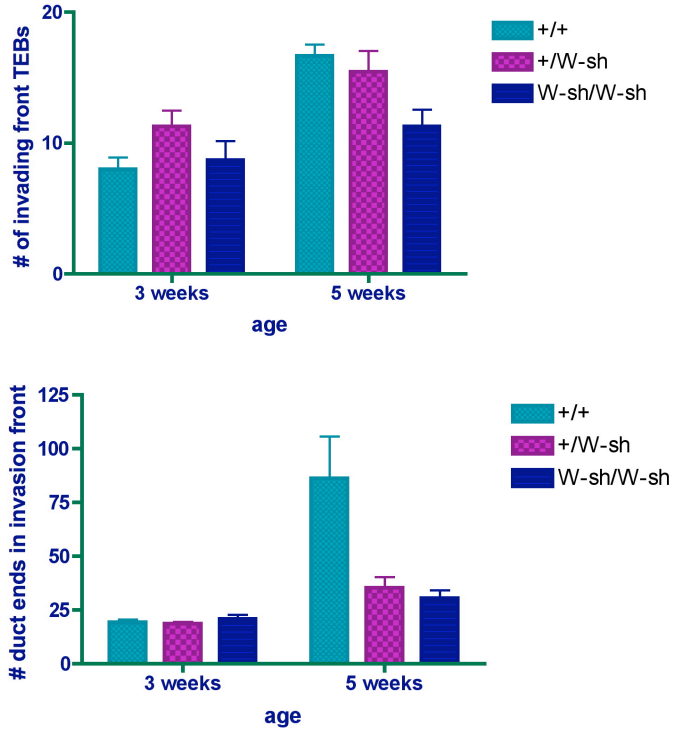

Figure 9. Mast cells affect duct invasion into the mammary fat pad at 5 weeks of age. Whole mounts of left inguinal mammary glands were collected and examined for duct length. Values are expressed as the mean of the three longest ducts originating at the nipple. ANOVA: p=0.0049.